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SIALYLTRANSFERASE ACTIVITIES OF AGING DIPLOID FIBROBLASTS

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Summary

Sialyltransferase activity and cell-cell adhesion rates of aging WI-38 cells were studied to determine the possible basis for a previously described decrease in membrane bound sialic acid and loss of proliferation of senescent cells. Ecto-sialyltransferase was demonstrated on the surface of both young and old WI-38 cells. The sialyltransferase assays consist of an enzyme source which is either the surface of intact cells (ectoenzyme) or a Triton X-100 cell homogenate, the nucleotide sialic acid donor (cytidine monophosphate-*N*-acetylneuraminic acid), and an asialo-acceptor which may be endogenous to the enzyme preparation or may be added exogenously. When sialyltransferase activity is measured in the absence of exogenous acceptors, there is a greater amount of sialic acid transferred by old cells. However, when exogenous acceptors are provided, the amount of transfer is stimulated to a greater extent in young cells equalizing the amount of sialic acid incorporated into young and old cells. This suggests that there are fewer asialoglycoproteins and that acceptor concentration is a limiting factor in assays of young cell sialyltransferase. The end result of this may be the previously described decreased amount of membrane-bound sialic acid of old cells. A change in the adhesiveness of old cells is described which may be related to the altered cell surface.

Introduction

Human diploid fibroblasts have a finite lifespan in vitro, and this cell system has been widely used as a model for studying the cellular expression of aging. Cristofalo and Sharf [1] have shown that as the replication of the cell population decreases with increasing in vitro passage, the proportion of the cells which

Abbreviation: CMP-AcNeu, CMP-*N*-acetylneuraminic acid.

are capable of cell division declines. A variety of biochemical changes that accompany the declining proliferating capacity of WI-38 cells have been described. One of the most well documented changes is an increase in lysosomal enzyme activity during in vitro aging [2,3]. Although there is good evidence that the plasma membrane of the cell plays a role in control of proliferation [4], there have been few reports characterizing the cell surface properties of aging cells [5—8].

Recent work has shown that the electrophoretic mobility, a measure of net cell surface charge, decreases as the in vitro passage time of WI-38 cells increases [3]. In many cell types [9] sialic acid has been found to be one of the main determinants of the net negative charge expressed at the cell surface; a decrease in membrane-bound sialic acid has been reported in senescent WI-38 cells [10]. A decrease in the surface charge and accelerated senescence of red blood cells following neuraminidase treatment has also been demonstrated [11]. Cell surface sialic acid has been implicated in cell-cell recognition and adhesion [12], contact inhibition [13], and immunogenicity [14]. It has been postulated that the enzymes which catalyze the transfer of terminal sialic acid residues onto cell surface glycoproteins play a major role in mediating cellular interactions in a variety of tissues and in vitro cell culture systems [15].

The purpose of these studies was to determine if changes in cell surface sialyltransferase activity are responsible for the membrane alterations previously demonstrated in senescent WI-38 cells [3,10] and to examine the relationship of any detectable changes in ectoenzyme activity to functional changes in the cell surface-mediated functions, cell recognition and adhesion.

Materials and Methods

Cell culture and cell aging. Monolayer cultures of WI-38 cells at various passage levels were originally obtained from Dr. Leonard Hayflick. The cells are grown and frozen in liquid N_2 according to the method described by Hayflick and Moorhead [16]. Routinely the cells are subcultured in basal minimal essential medium containing 10% calf serum (Gibco) at a split ratio of 2 : 1 every 3—4 days. As the cells approach the end of their lifetime in culture, the time between subculturing is increased to allow the cell to become confluent. When the cells fail to reach confluency after one week, the cultures are terminated. Cells which fail to reach confluency are not used experimentally. The age of the WI-38 cells used in these studies was determined by the method of Cristofalo and Sharf [1]. Cell size as an indication of cell age [17] and the growth properties of the cells were monitored using a Coulter Counter Model ZB with an attached Channelyzer.

Ectotransferase assay. Cell surface sialyltransferase activity was assayed as previously described, with minor modifications [18]. Intact single cells were obtained from confluent monolayers growing on Corning plastic roller bottles by incubating the cells in phosphate-buffered saline containing 1 mM EDTA for 6 min at 37°C. Cells were collected by centrifugation for 5 min at 500 × *g* and then washed two times in Earle's phosphate-buffered balanced salt solution containing 0.1% glucose. Cell number and average cell size were determined by Counter electronic particle counting. To assay ectosialyltransferase activity,

total cell protein, rather than cell number, was kept constant. Approximately 0.35 mg of protein, as determined by the Lowry method [19], was incubated with 1.5 μM CMP-*N*[^{14}C]acetylneuraminic acid (CMP-AcNeu; New England Nuclear; specific activity 230 Ci/mol unless otherwise specified) in Earle's phosphate-buffered salt and glucose solution (pH 6.8). Addition of 2.5 mM $MgCl_2$ and 2.5 mM $MnCl_2$ gave maximum enzyme activity for both young and old cells; omission of divalent cation resulted in only 75—80% of this activity. UTP (final concentration 1 mM) was added to prevent hydrolysis of the CMP-[^{14}C]AcNeu [20]. The cells were incubated in a final volume of 80 μl for various times at 37° C. The reaction was terminated by the precipitation of cell protein with 1.0% phosphotungstic acid in 0.4 N HCl followed by two washes with 10% trichloroacetic acid and extraction of glycolipids with ethanol/ether (2 : 1). The final pellet was dissolved in 1.0 N NaOH and dried on glass fiber filters; radioactivity incorporated into glycoproteins was determined by liquid scintillation spectrometry. In some instances the enzymatic reaction was terminated by the addition of 200 μl of 95% ethanol; the ethanol extract was then chromatographed to monitor hydrolysis of the CMP-[^{14}C]AcNeu. The viability of the cells was determined by trypan blue exclusion. Only cell populations with greater than 90% viability were used to assay whole cell ecto-transferase activity.

Attempts to stimulate ectoenzyme activity by increasing the number of acceptors available for the transfer of sialic acid included (1) the addition of asialofetuin, prepared as previously described [21] and (2) pretreatment of the cells with increasing concentrations of neuraminidase (*Clostridium perfringens*) in a balanced salt solution (pH 6.8) for 5 min at 37° C. The cells were then washed twice and assayed for ectosialyltransferase activity.

Total sialyltransferase activity. Total enzyme activity is defined as the sialyl-transferase measured in 0.1% Triton X-100/0.1 M Tris (pH 7.0) homogenates of young and senescent WI-38 cells. The incubation medium contained 2.5 mM $MnCl_2$, 2.5 mM $MgCl_2$, and 1.0 mM UTP. Acid-insoluble radioactivity was determined as described previously. Exogenous transferase activity was measured by the addition of 300 μg of asialofetuin.

Cell adhesion assay. The assay used was a modification of the method described by Walther et al. [22]. Single cells were labeled by incubation with a ^{3}H-labeled amino acid mixture for 48 h followed by a 3 h incubation in fresh medium. Single cells were prepared by treatment with 0.25% trypsin for 1 min. The trypsin solution was then removed and the cells were incubated for 10 min at 27° C. Basal minimal essential medium containing 10% calf serum was added, and the cells were removed from the plastic flasks by gentle shaking. The cells were washed once and then diluted with medium minus serum to a final concentration of $2 \cdot 10^4$ cells/ml. 5.0 ml of cells were added to monolayers of young and old cells in 25-cm^2 plastic Falcon flasks in which the monolayers were microscopically confirmed to be confluent. The incubations were conducted at 37° C on a rocking platform. After incubation, the cells in suspension were removed by aspiration and subsequently the monolayers were washed twice with medium minus serum. The monolayers were solubilized with 2.0 ml of 1.0 M NH_4OH and 1.0 ml of the extract was counted in 10.0 ml of toluene/Triton X-100. Cpm/cell were calculated from an acid precipitate; over 99% of the radioactivity in the cells was acid precipitable.

Results

Ectosialyltransferase assay

When intact, viable WI-38 cells, both young and senescent, were incubated with CMP-[^{14}C]AcNeu, radioactivity was incorporated into cell surface glycoproteins. For these studies, cells between passages 18 and 23 were considered to be young, and fibroblasts that had reached passages 40—45 were classified as senescent according to the previously described procedures. Fig. 1 indicates that the rate of incorporation of CMP-[^{14}C]AcNeu is linear for 15 min under the conditions of the assay and that old cells have a higher rate of transfer of AcNeu onto endogenous acceptors.

Cell surface location

Since ectoglycosyltransferases have not been previously demonstrated in normal human cells, several lines of evidence were obtained to document ectoenzyme activity. The ability of the intact cells to exclude trypan blue for the short incubation times was greater than 90%. It is generally accepted that intact, viable cells are impermeable to CMP-[^{14}C]AcNeu. However, a portion of the nucleotide sugar is hydrolyzed, and if free sialic acid entered the cell, it might be reactivated to CMP-AcNeu and utilized by sialyltransferases inside the cell. This possibility was eliminated by the fact that addition of 1000-fold excess of unlabeled sialic acid did not reduce incorporation of [^{14}C]AcNeu. Although WI-38 cells do hydrolyze CMP-[^{14}C]AcNeu, degradation of the substrate by both young and old cells was decreased to less than 5% by the addition of UTP at a final concentration of 1.0 mM to the assay medium. The experimental

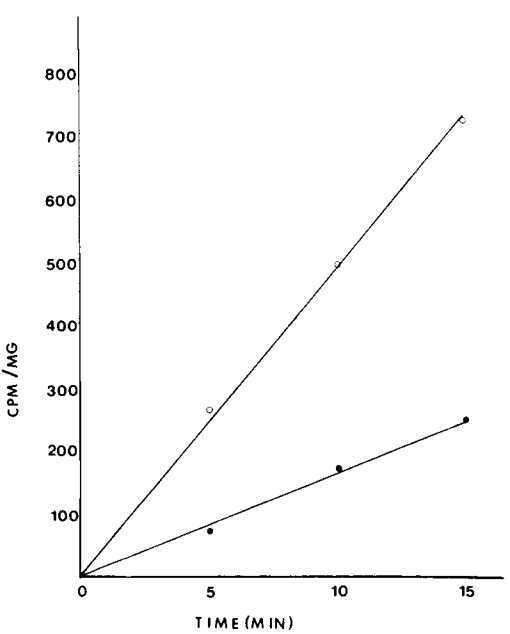

Fig. 1. Kinetics of incorporation of [^{14}C]AcNeu into acid-insoluble endogenous acceptor protein. Enzyme activity was assayed as described in Materials and Methods. ○, senescent cells; ●, young cells.

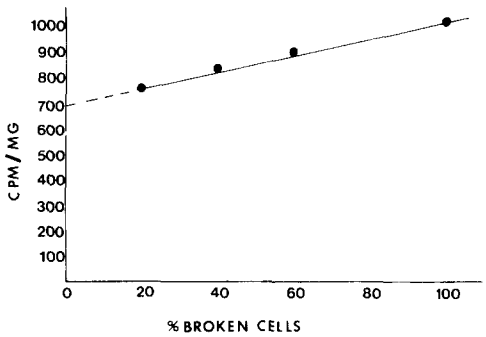

Fig. 2. Ectotransferase activity of passage 40 WI-38 cells. Intact cells were broken by repeated freezing and thawing. Various percentages of broken and whole cells (final protein concentration remained constant) were incubated for 15 min at 37°C and the amount of acid-insoluble radioactivity was determined by liquid scintillation spectrometry as described in Materials and Methods.

results illustrated in Fig. 2 prove that the enzyme activity measured was not due to a few broken cells in the assay. According to the method of Struck and Lennarz [23], the addition of up to 100% broken cells to the assay while total protein remained constant did not greatly increase incorporation; and when enzyme activity was plotted against percent broken cells, extrapolation of the graph to 0% broken cells indicated an amount of AcNeu incorporation corresponding to that measured by the ectosialyltransferase.

Total sialyltransferase activity

When 0.1% Triton X-100/0.1 M Tris (pH 7.0) homogenates of young and senescent cells were incubated with CMP-[^{14}C]AcNeu, as seen in Table I, the difference in whole cell enzyme activity was similar to that seen with the ectosialyltransferase; the old cells demonstrated substantially higher levels of transferase activity. When asialofetuin was added to the incubation medium, activity in the young cells was stimulated 5—7-fold; transferase activity in senescent cells was not stimulated to the same extent (Table I). The result of the greater stimulation in young cell enzyme activity was to equalize the amount of [^{14}C]-

TABLE I

TOTAL SIALYLTRANSFERASE LEVELS

Total sialyltransferase activity was assayed as described in Materials and Methods. Exogenous enzyme activity was determined by subtracting the amount of incorporation which occurs without added acceptor from the results obtained when asialofetuin is included in the incubation medium.

Incubation condition	Sialyltransferase activity (cpm/mg per h)	
	Young cells	Senescent cells
Minus acceptor	1690 ± 1000 *	4560 ± 1400
Plus acceptor	7800 ± 1980	6440 ± 1800
Exogeneous activity	6110	1880

* Means ± 1 S.D. of four separate experiments.

AcNeu transferred to both endogenous and exogenous acceptors in the young and senescent cells. This finding suggested that endogenous acceptor concentration rather than enzyme activity might be the limiting factor in the in vitro assay of sialyltransferase in young WI-38 cells. Since asialofetuin was not a good substrate for the ectoenzymes, the possibility of stimulating ectoenzyme activity in young cells by increasing endogenous acceptors was investigated.

Exogenous acceptors for ectosialyltransferase

As seen in Table II when desialylated fetuin was included in the assay medium, the ectosialyltransferase of young WI-38 cells transferred [^{14}C]AcNeu to this exogenous acceptor. Although the mean increase in radioactivity incorporated was not statistically different, there was a consistent percentage increase over four separate experiments. Sialyltransferase activity in young cells was always stimulated to a limited extent by the addition of exogenous acceptor, but no increase in activity was observed when asialofetuin was included in the assay of senescent cell activity. The results were calculated on a basis of both per cell and per mg of protein, since the amount of protein/cell was found to be almost double at senescence.

Alternatively, we attempted to augment the endogenous population of acceptor molecules on the cell surface by removal of cell surface sialic acid. By incubating the cells with increasing concentrations of neuraminidase, it was possible to generate a dose-dependent increase in the amount of [^{14}C]AcNeu transferred to glycoprotein in both the young and the old cells (Table III). It can be seen from Table III that after neuraminidase treatment young cells show a higher percent increase in AcNeu transferred. After treatment with 0.16 unit of neuraminidase, there is no significant difference in the amount of [^{14}C]-AcNeu incorporated into cell surface glycoproteins of young and senescent cells.

Kinetics of cell adhesion

Since a correlation between the ectoglycosyltransferase system and cell-cell adhesiveness has been postulated, we measured the adhesion of young and old

TABLE II

ECTOSIALYLTRANSFERASE ACTIVITY

Intact cells were incubated under the conditions described (plus or minus asialofetuin) for 15 min. Cell protein was measured by the Lowry method. Cell numbers were determined with a Coulter Counter (Model ZB). Expressing the data on a per cell basis increased the difference in enzyme activity between young and old cells since the senescent cells (passages 40—45) have approximately twice as much protein as the young cells (passages 18—23). Values are means of four separate experiments (with 1 S.D. for the per mg data).

Incubation condition	Young cells		Senescent cells	
	cpm/mg per h	cpm/10^6 cells per h	cpm/mg per h	cpm/10^6 cells per h
Minus acceptor	1162 ± 411	332	3003 ± 100	1668
Plus acceptor	1529 ± 100	437	3000 ± 250	1666
Exogenous activity		105		0

TABLE III

STIMULATION OF ECTOSIALYLTRANSFERASE ACTIVITY BY PRETREATMENT WITH NEURAMINIDASE

(Cells were incubated with neuraminidase (*Cl. perfringens*) for 5 min at 37° C and washed twice prior to ectosialyltransferase assay.

Units of neuraminidase	Young cells		Old cells	
	cpm/mg per h	% control	cpm/mg per h	% control
0	1216 ± 391 *	0	3174 ± 150	0
0.04	3487 ± 324	287	4277 ± 1049	135
0.08	4532 ± 212	373	5891 ± 862	186
0.16	7210 ± 981	593	8104 ± 1002	255

* Results are cpm/mg protein per h ± 1 S.D. for four separate experiments.

cells to monolayers of both young and old WI-38 cells. Fig. 3 depicts the time course of adhesion of labeled single cells to confluent monolayers. Cell attachment at 37° C was rapid for the first 15 min and then proceeded more slowly. The single-cell nature of the suspension was verified by microscopy and by using a Coulter electronic sizing device. The fact that single cells rather than aggregates were adhering to the monolayers was determined microscopically. The rate of attachment did not vary significantly with the age of the monolayer, but it is evident from Fig. 3 that the rate of attachment of young cells to confluent monolayers proceeds more rapidly than the attachment of senescent

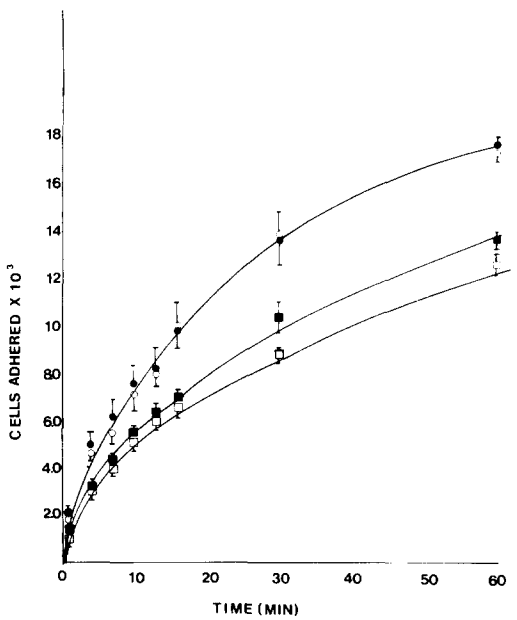

Fig. 3. Cellular adhesion of aging WI-38 cells. The assay is described in Materials and Methods. Results are the means ±1 S.D. for three separate experiments. The rate of attachment of young cells to young monolayers (●) and to old monolayers (○) and the attachment of old cells to young monolayers (■) and old monolayers (□) was measured.

cells to young and old cell monolayers. Since it was necessary to use trypsin in order to prepare single-cell suspensions, the possibility exists that differences in sensitivity to trypsin damage and repair rate might result in differences in the rate of cell attachment. Using the galactose oxidase-borohydride method for labeling the cell surface, we have been unable to detect any differences in rate of repair of cell surface galactose or sialic acid residues for periods up to 1 h after trypsinization, the duration of the adhesion assay (Myers-Robfogel, M., Bosmann, H.B., Spataro, A.C., unpublished results). However, trypsin damage (i.e. removal of certain glycopeptides) may be different in young and old cells. This possibility is suggested by the fact that old and young monolayers exhibit similar adhesiveness but dissociated young and old cells do not.

Discussion

As WI-38 cells age in vitro they gradually lose the potential to divide [1]. It is conceivable that modifications of the cell surface membrane could impair cell division since a relationship between the structure and function of the plasma membrane and cell division has been demonstrated in differentiating and transformed cells [24]. The purpose of this investigation was to determine if a relationship exists in aging human cells between the activity of a cell surface enzyme ectosialyltransferase, plasma membrane sialic acid content, and cellular adhesiveness. Good evidence for the existence of ectosialyltransferase has been obtained using Ehrlich ascites tumor cells [25], murine leukemic cells [26], and mouse thymocytes [27]. Since ectosialyltransferase had not previously been demonstrated in normal human fibroblasts, a number of experiments were performed to prove the cell surface location of the enzyme. The following facts argue for a cell surface location for the transferase when intact, viable WI-38 cells are incubated with CMP-[^{14}C]AcNeu: (1) substrate hydrolysis to CMP and [^{14}C]AcNeu was limited to 5%; (2) inclusion of 1000-fold excess of unlabeled AcNeu did not inhibit incorporation of [^{14}C]AcNeu from CMP-[^{14}C]AcNeu; and (3) increasing percentages of artificially broken cells did not markedly affect the amount of radioactivity incorporated and when extrapolated to zero percent broken cells, enzyme activity is identical to that obtained in the ectoenzyme assay.

The results presented show that under the conditions of the ectosialyltransferase assay using endogenous cell surface glycoproteins as acceptors for *N*-[^{14}C]acetylneuraminic acid, enzyme activity in the senescent cells appears to be elevated. Since differences in sialyltransferase activity have been directly related to cellular sialic acid levels in many tissue culture cell lines [28], the finding that senescent WI-38 cells had an apparent increase in ectosialyltransferase activity while membrane-bound sialic acid [10] and cell surface charge decreased [3], was surprising and suggested that either the conditions of the in vitro ectoenzyme assay were not simulating in vivo cell culture conditions or that the function of the assayed ectosialyltransferase is not primarily the synthesis of cell surface sialylglycoproteins. One possible explanation for the difference in enzyme activity detected in vitro was a change with developing senescence in acceptor concentration at the cell surface. To study this possibility, excess exogenous acceptor asialofetuin was added to the whole cell incuba-

tions. There was a small stimulation of the enzyme activity associated with the cell surface of young cells but no stimulation of activity in senescent cells. Since the exogenous acceptor was not readily utilized by the ectoenzymes, although it stimulated transferase activity in Triton X-100 homogenates, it was not possible to determine conclusively the cell surface acceptor concentration from these experiments. A similar behavior of ectosialyltransferase toward exogenous acceptors has been previously described [29].

It has been demonstrated that pretreating cells with neuraminidase will increase the number of free galactose residues available for the transfer of *N*-[^{14}C]acetylneuraminic acid and thereby increase the amount of measurable radioactivity incorporated [18,30]. By pretreating the young and old cells with increasing concentrations of neuraminidase it was possible to decrease the apparent difference in ectosialyltransferase activity. This suggested again that acceptor concentration was the limiting factor in the assay of ectosialyltransferase activity in young cells and that senescent cells have free or unsialylated galactose residues on their cell surfaces. Further proof that senescent cells do have an increase in terminal cell surface galactose residues has been obtained [31], and this is thought to be the explanation for the apparent increase in senescent cells of ectosialyltransferase activity when the enzyme is assayed in the absence of exogenous acceptors. Possible explanations for the decrease in cell surface sialic acid of senescent cells which we are currently investigating include an increase in neuraminidase activity or a decrease in the substrate, CMP-*N*-acetylneuraminic acid.

The origin and relationship of ectotransferases to the glycosyltransferases found in the Golgi apparatus are still unclear. Since a difference in the behavior of the ectosialyltransferase during aging had been found, the question of whether total cell activity might be similarly affected was asked with one possible result being that the activity of the cell surface sialyltransferase could be differentiated from the Golgi enzyme. It can be seen from the results, that a large fraction of the endogenous cell homogenate sialyltransferase activity is expressed at the cell surface, but the activity present in cellular homogenates, unlike that at the cell surface, can be stimulated several fold by the addition of exogenous asialofetuin. The same relative differences were found to exist in total sialyltransferase activity between young and senescent cells, but the addition of excess asialofetuin caused a greater stimulation of transferase activity in the young cells and effectively equalized the amount of *N*-[^{14}C]acetylneuraminic acid incorporated into glycoproteins by young and old cells. This result suggests that, unless some other factors, such as ion or substrate concentrations, are limiting activity in the assay of total cell sialyltransferase in senescent cells, sialyltransferase activity decreases as cells age in vitro. Mitsui and Schneider [32] have shown that the small subpopulation of dividing cells in a senescent culture has different properties from the subpopulation of dividing cells in young cultures. This suggests that any differences detected between young and senescent populations are due not solely to the percentage of replicating cells in the population because replicating cells of old cultures maintain their 'age' upon further culture.

The question of what functional role ectotransferases have seems to be less well established than the fact that they do exist [15]. The present studies show

that cell-to-cell adhesiveness decreases as cells age. Similar results have been obtained with aging chick cells maintained in vitro [7]. Other workers have correlated increased cellular aggregation of malignant rat fibroblasts with an increase in acceptor sites for sialyl- and galactosyltransferases [33]. We are currently investigating whether trypsinization affects old and young cell surfaces differently, thereby altering measured adhesiveness. Since the study of the development of senescence of WI-38 cells routinely uses trypsin for subculturing the cells, the effects of trypsin on the ability of cells to reattach to the substratum after enzyme disaggregation may be significant to the study of cellular aging in vitro.

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